Annals of Applied Biology ISSN 0003-4746

RESEARCH ARTICLE

Lysine and threonine biosynthesis in sorghum seeds: characterisation of aspartate kinase and homoserine dehydrogenase isoenzymes

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Keywords
Amino acids; aspartate kinase; cereal crops; lysine; nitrogen; nutritional balance; threonine.

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Received: 2 May 2006; revised version accepted: 31 May 2006.

doi:10.1111/j.1744-7348.2006.00074.x

Abstract

Aspartate kinase (AK, EC 2.7.2.4) and homoserine dehydrogenase (HSDH, EC 1.1.1.3) have been partially purified and characterised from immature sorghum seeds. Two peaks of AK activity were eluted by anion-exchange chromatography [diethylaminoethyl (DEAE)-Sephacel] with 183 and 262 mM KCl, and both activities were inhibited by lysine. Similarly, two peaks of HSDH activity were eluted with 145 and 183 mM KCl; the enzyme activity in the first peak in elution order was shown to be resistant to threonine inhibition, whereas the second was sensitive to threonine inhibition. However, following gel filtration chromatography (Sephacryl S-200), one peak of AK activity co-eluted with HSDH and both activities were sensitive to threonine inhibition, suggesting the presence of a bifunctional threonine-sensitive AK–HSDH isoenzyme with a molecular mass estimated as 167 kDa. The activities of AK and HSDH were studied in the presence of lysine, threonine, methionine, valine, calcium, ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′*N*′-tetraacetic acid, calmodulin, S-adenosylmethionine (SAM), S-2-aminoethyl-L-cysteine (AEC) and increasing concentrations of KCl. AK was shown to be inhibited by threonine and lysine, confirming the existence of two isoenzymes, one sensitive to threonine and the other sensitive to lysine, the latter being predominant in sorghum seeds. Methionine, SAM plus lysine and AEC also inhibited AK activity; however, increasing KCl concentrations and calcium did not produce any significant effect on AK activity, indicating that calcium does not play a role in AK regulation in sorghum seeds. HSDH also exhibited some inhibition by threonine, but the majority of the activity was not inhibited, thus indicating the existence of a threonine-sensitive isoenzyme and a second predominant threonine-insensitive isoenzyme. Valine and SAM plus threonine also inhibited HSDH; however, increasing concentrations of KCl and calcium had no inhibitory effect.

Introduction

Amino acids are key nitrogen-containing compounds and protein constituents, and their metabolism is a fundamental process to plant growth and development (Andrews *et al.*, 2004; Medici *et al.*, 2004*a*; Raven *et al.*, 2005). Amino acid and nitrogen metabolism studies are essential in order to improve our understanding of several distinct aspects of general plant metabolism (Medici *et al.*, 2004*b*; Wilman *et al.*, 2004; Elgersma *et al.*, 2005; Trethowan *et al.*, 2005). Amino acid metabolism in plants is controlled by a complex regulatory network involving a large number of enzymes and intermediates (Lee *et al.*, 2005; Azevedo *et al.*, 2006). Lysine, methionine, isoleucine and threonine are essential amino acids that are present in limiting amounts in the seeds of many crop plants and are considered to be of great nutritional importance in animal feedstuffs and human food (Galili *et al.*, 2002; Nikiforova *et al.*, 2006; Stepansky *et al.*, 2006). These essential amino acids are synthesised

in plants via different branches of a pathway that commences with aspartate (Azevedo *et al.*, 1997).

In developing countries, cereal crop seeds are the main dietary source of protein for human and livestock (Helm *et al.*, 2004). Among the cereal crops, sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important, being the major source of protein for millions of people (mainly in Africa) and for livestock worldwide. However, the protein quality of sorghum is low as a result of an imbalance in essential amino acids in the seed storage proteins in a manner similar to that of other cereal crops (Fornazier *et al.*, 2005; Landry *et al.*, 2005).

A major goal of plant science for many years has been the development of cereal crops with higher amounts of lysine and threonine in the seeds (Ferreira *et al.*, 2005*a*). In order to achieve such a goal, the aspartate metabolic pathway has been investigated in detail, and important regulatory steps have been identified (Hudson *et al.*, 2005; Azevedo *et al.*, 2006; Pompeu *et al.*, 2006) (Fig. 1). Some key enzymes of the aspartate pathway such as aspartate kinase (AK, EC 2.7.2.4), homoserine dehydrogenase (HSDH, EC 1.1.1.3), dihydrodipicolinate synthase (EC 4.1.2.52) and threonine synthase (EC 4.2.99.2) have been isolated, purified and characterised in several plant species (Azevedo *et al.*, 2006). Most have been shown to be present in different isoenzymic forms and under the control of a number of genes (Vauterin *et al.*, 1999; Rognes *et al.*, 2003; Curien *et al.*, 2005).

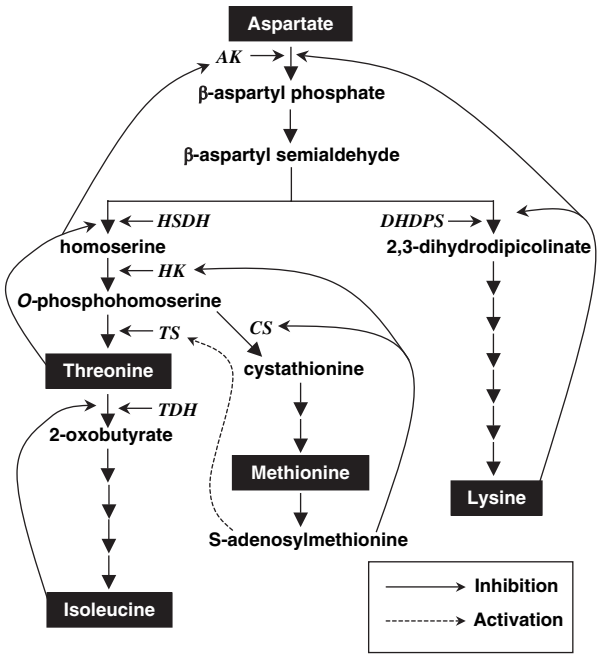

Figure 1 The aspartate metabolic pathway leading to the synthesis of lysine, threonine, methionine and isoleucine. Enzymes: AK, aspartate kinase; CS, cystathionine γ-synthase; DHDPS, dihydrodipicolinate synthase; HK, homoserine kinase; HSDH, homoserine dehydrogenase; TDH, threonine dehydratase; TS, threonine synthase. Feedback inhibition and activation by the amino acids end products are indicated.

AK catalyses the phosphorylation of aspartate to form β-aspartyl phosphate, and up to three AK isoenzymes have been observed in plants, which are subject to feedback inhibition, either by lysine or by threonine (Azevedo *et al.*, 1997). The lysine-sensitive form of AK, which may also be synergistically feedback inhibited by a combination of lysine and S-adenosylmethionine (SAM), is normally predominant in plant tissues, accounting for approximately 50–70% of the total AK activity, whereas the threonine-sensitive AK isoenzyme normally accounts for approximately 20% of the total AK activity (Azevedo *et al.*, 2006).

In a branch of the pathway, aspartate semialdehyde is reduced to homoserine in a reaction catalysed by the enzyme HSDH, which uses reduced nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate (Azevedo *et al.*, 1997). Two isoenzymes, one sensitive to threonine feedback inhibition and another resistant to threonine inhibition, have been observed in plants (Azevedo *et al.*, 2006). The AK and HSDH isoenzymes sensitive to threonine feedback inhibition have been shown to be part of a single bifunctional polypeptide (Azevedo *et al.*, 2006).

As far as we are aware, until two very recent reports by our group, lysine metabolism had not been previously investigated in sorghum. We have recently isolated lysine 2-oxoglutarate reductase (LOR, EC 1.5.1.8) and saccharopine dehydrogenase (EC 1.5.1.9), which are involved in lysine degradation, in sorghum seeds (Fornazier *et al.*, 2005), but only preliminary results have been reported for AK and HSDH from sorghum seeds. In this work, we present the partial purification and characterisation of AK and HSDH from sorghum seeds, and some regulatory aspects have also been investigated and a comparison with other cereal crop enzymes is also presented.

Materials and methods

Plant material

The commercial hybrid of sorghum (*Sorghum bicolor*) 'Massa 03' was used throughout. Plants were grown in the field of the experimental station of the Departamento de Genética, ESALQ-USP, Piracicaba, Brazil, during the summer season of 2001–2002, and immature sorghum seeds at the milky stage (97 days of growth) were harvested, immediately frozen into liquid nitrogen and stored at −70°C until used.

Enzyme extraction

Extraction of AK and HSDH was carried out at 4°C exactly as described by Ferreira *et al.* (2005*b*), with minor

modifications. Immature sorghum seeds were used and extracted in 5 volumes of buffer A (50 mM Tris–HCl, pH 7.4, containing 200 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 2 mM L-lysine, 2 mM L-threonine, 10% (v/v) glycerol and 5% (w/v) polyvinylpolypyrrolidone. The extract was filtered through five layers of gauze and centrifuged at 16 000 *g* for 30 min to remove completely the cell debris from the extract.

Ammonium sulphate precipitation

AK and HSDH enzymes were precipitated with 20–60% saturation of solid ammonium sulphate according to Lugli *et al.* (2002). The precipitated protein was recovered by centrifugation at 16 000 *g* for 30 min, and the protein pellet was dissolved in a small volume of buffer B [25 mM Tris–HCl, pH 7.4, 1 mM DTT, 0.1 mM L-lysine, 0.1 mM L-threonine and 10% (v/v) glycerol], loaded onto a Sephadex G25 column (2.5 × 20 cm) equilibrated with 5 volumes of buffer B and run under gravity.

Enzyme purification

The desalted sample was loaded onto a diethylaminoethyl (DEAE)-Sephacel column (2.5 × 12 cm; flow rate 1 mL min^{-1}) equilibrated in buffer B and connected to a chromatography Bio-Rad Econo System. The column was washed extensively with buffer B and the unbound fraction stored at −20°C for further analysis. The column was then developed by a linear gradient from zero to 500 mM KCl (200 mL total volume) at 1 mL min^{-1}. Fractions of 2 mL were collected and used for AK and HSDH activity determination. Fractions containing AK and HSDH activities were pooled and concentrated with 70% saturated ammonium sulphate. After stirring for 1 h, the sample was centrifuged at 16 000 *g* for 30 min, and the precipitated protein was dissolved in buffer B and 5 mL of the redissolved sample was applied to a Sephacryl S-200 gel filtration column (2.6 × 83 cm) equilibrated in the same buffer. The column was developed with buffer B at 0.5 mL min^{-1}, and fractions of 2 mL were collected and assayed for AK and HSDH activities. The full purification procedure was repeated several times over a period of 2 years, and the data presented are a representative sample of one such purification.

AK assay

AK was assayed by a modified hydroxamate method in a final volume of 500 μL as described by Azevedo *et al.* (2003). The assay mixture comprised 100 μL of 100 mM Tris–HCl (pH 7.4) containing 1 mM DTT and 20% (v/v) glycerol, 100 μL of 500 mM aspartic acid (pH 7.4), 50 μL of 125 mM magnesium sulphate, 50 μL of 200 mM adenosine triphosphate (ATP) (pH 7.4), 50 μL of 4 M hydroxylamine (pH 7.4), 100 μL of H_2O and 50 μL of enzyme extract. The reaction was initiated by the addition of ATP and the assay mixture was incubated at 35°C for 10–30 min. After this period, the assay was stopped by the addition of 500 μL of ferric chloride reagent [0.67 M $FeCl_3$, 0.37 M HCl and 20% (w/v) trichloroacetic acid]. After the removal of precipitated protein by centrifugation at 10 000 *g* for 10 min, the absorbance of the supernatant was read at 505 nm. Controls containing lysine and threonine were normally included to ensure that the activity measured was due to AK and to identify the isoenzymes inhibited by the two amino acids.

HSDH assay

HSDH was assayed spectrophotometrically at 340 nm at 30°C in a final volume of 1.0 mL as described by Azevedo *et al.* (2003). The assay mixture comprised 700 μL of 100 mM Tris–HCl (pH 9.0) containing 150 mM KCl, 1 mM DTT and 0.5 mM EDTA, 100 μL of 200 mM DL-homoserine, 100 μL of 4.8 mM NADP, 50 μL of H_2O and 50 μL of enzyme. The effect of threonine was determined by the addition of the amino acid to the HSDH assay mixture.

Effect of calcium, calmodulin inhibitors and ionic strength on AK and HSDH activities

AK and HSDH activities were determined in a sample representing a pool of the DEAE-Sephacel first peak in elution order in the presence of 1.6 mM calcium ($CaCl_2$), 1.6 mM ethylene glycol bis(2-aminoethylether)-*N*, *N*, *N′N′*-tetraacetic acid (EGTA), 1.6 mM calcium plus 1.6 mM EGTA, 10 μg/mL calmodulin, 1.6 mM calcium plus 10 μg mL^{-1} calmodulin and 50 μg mL^{-1} compound 48/80 and in the presence of salt of varying concentrations ranging between 0, 100, 200, 300, 400 and 500 mM KCl. Blanks with and without the compounds being tested were included. Negative controls lacking the substrates, aspartic acid and homoserine, were also assayed on each occasion.

Effects of amino acids and analogues on AK and HSDH activities

AK and HSDH total activities were determined in the presence of 1–5 mM L-lysine, 1–5 mM L-threonine, 1–5 mM L-lysine plus 1–5 mM L-threonine, 1–5 mM methionine, 1–5 mM valine, 1–5 mM S-2-aminoethyl-L-cysteine

(AEC), 1 mM SAM, 1 mM SAM plus 5 mM threonine and 1 mM SAM plus 5 mM lysine. Blanks and negative controls were tested as described above.

Molecular mass determination

The molecular mass of AK was determined by gel filtration chromatography on a Sephacryl S-200 column (2.6 × 86 cm). The determination was carried out simultaneously with the purification of AK as described above. The Sephacryl S-200 column was calibrated by the use of the following molecular mass markers: blue dextran to determine the void volume of the column (2000 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa) and horse heart cytochrome *c* (12.4 kDa).

Protein determination

The protein concentration of all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Results

AK partial purification

The results of the AK purification procedure are shown in Table 1. The AK purification procedure resulted in 29.9-fold purification to a final yield of 8.2% of total AK activity. Two peaks of AK activity were eluted from the DEAE-Sephacel column with a 0–500 mM KCl linear gradient (Fig. 2A). The first and the second peaks in elution order were eluted with approximately 183 and 286 mM KCl, respectively. The AK activities in both peaks were shown to be inhibited partially by lysine and therefore were designated as AK-LysI and AK-LysII, respectively. However, the first peak in elution order also exhibited some inhibition by threonine, and when both amino acids, lysine and threonine, were added together, the peak was almost totally inhibited. For HSDH, two overlapping peaks of activity were eluted from the DEAE-Sephacel column at approximately 145 and 183 mM KCl, respectively (Fig. 2B). The first HSDH peak of activity in elution order was shown to be resistant to inhibition by threonine and was designated as HSDH-R, whereas the second HSDH peak of activity was shown to be sensitive to inhibition by this amino acid. Since the second HSDH peak of activity co-eluted with the AK-LysI, which was also partially inhibited by threonine, the 183 mM HSDH peak was designated AK/HSDH-Thr.

Similarly, AK activity was eluted as two peaks from the Sephacryl S-200 gel filtration column. The enzyme activity in the first peak in elution order was shown to be strongly inhibited by threonine, whereas that in the second peak was shown to be inhibited by lysine (Fig. 3A). HSDH activity was also determined, and only one peak of activity that could be partially inhibited by threonine was identified, which co-eluted with the threonine-sensitive AK peak (Fig. 3B).

Molecular mass determination

The molecular mass of the AK proteins was determined by gel filtration chromatography (Sephacryl S-200). The first peak of threonine-sensitive AK enzyme was estimated to have a molecular mass of 167 kDa, whereas the enzyme in the second minor peak in elution order was estimated to have a molecular mass 79 kDa. The threonine-sensitive HSDH activity peak co-eluted with the threonine-sensitive AK peak, indicating a similar molecular mass of 167 kDa.

Effects of amino acids and analogues on AK and HSDH activities

The AK and HSDH activities of a combined mixture of fractions of the first peak in elution order from the anion-exchange chromatography linear gradient were analysed in the presence of lysine, threonine, methionine, valine, AEC and SAM. Since the purification procedure did not allow a complete separation of the two peaks

Table 1 Protocol for purification of aspartate kinase (AK) from 500 g of frozen immature sorghum seeds. Sequential steps of AK purification from one of several preparations

	Aspartate kinase				
Fraction	Total protein (mg)	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1} mg^{-1} protein)	Yield (%)	Purification factor (fold)
Crude extract	1890.3	—	—	—	—
20–60% ammonium sulphate	673.3	1131.3	1.7	100	—
DEAE-Sephacel (0–500 mM KCl)	154.21	3379.1	21.9	298.7	13
Sephacryl S-200 (combined isoenzymes)	1.9	92.9	50.2	8.2	29.9

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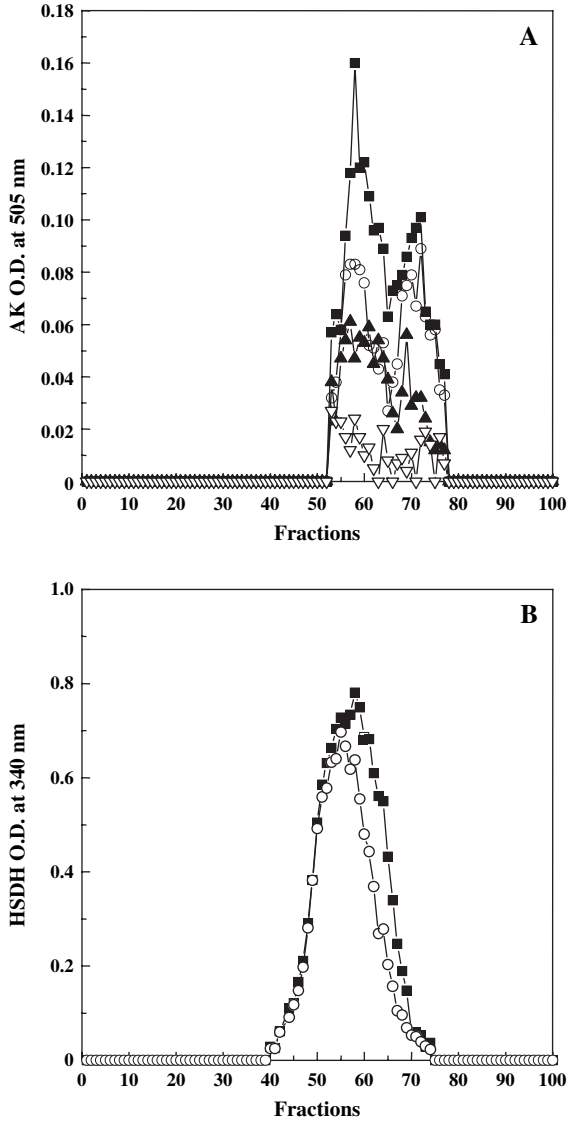

Figure 2 Elution profile of aspartate kinase (AK) (A) and homoserine dehydrogenase (HSDH) (B) isoenzymes isolated from sorghum seeds, from an anion-exchange chromatography column, using increased gradient concentrations of 0–500 mM KCl. (■) control, (○) threonine, (▲) lysine and (▽) lysine plus threonine treatments.

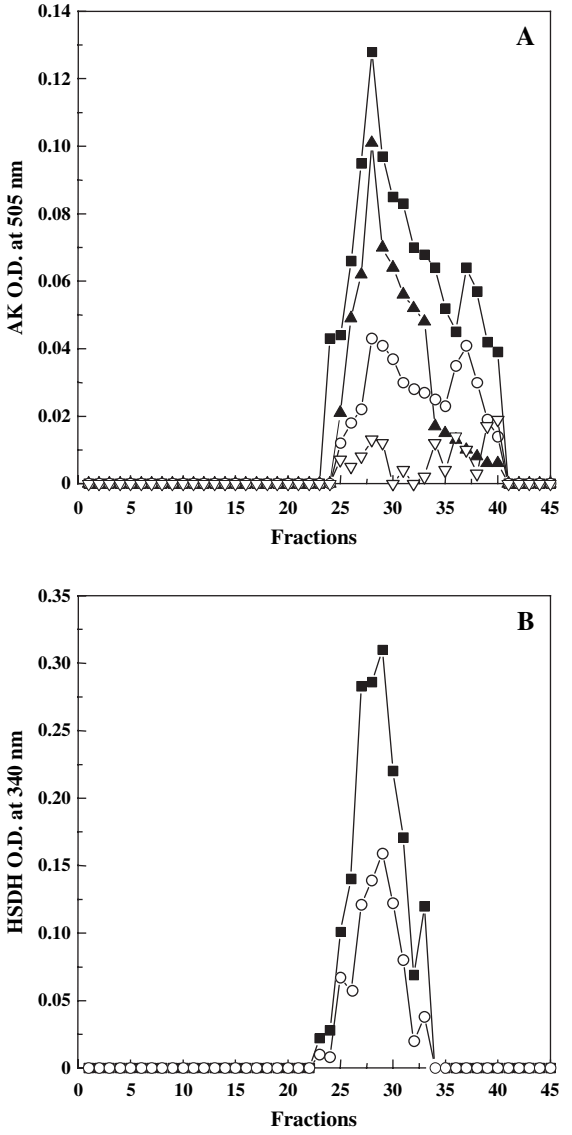

Figure 3 Elution profile of aspartate kinase (AK) (A) and homoserine dehydrogenase (HSDH) (B) isoenzymes isolated from sorghum seeds, from a gel filtration chromatography column Sephacryl S-200. (■) control, (○) threonine, (▲) lysine and (▽) lysine plus threonine treatments.

eluted from the DEAE-Sephacel column and subsequent purification resulted in considerable loss of enzyme activity, the predominant peak containing both enzyme activities was further characterised in relation to a number of compounds (Figs 4 and 5). Lysine and threonine were shown to be able to inhibit AK activity (Fig. 4A). Moreover, when both lysine and threonine were added together to the assay mixture, the strongest inhibitory effect on AK activity among all compounds and combinations tested was observed (Fig. 4A). AK activity was also inhibited by methionine at 5 mM, while valine did not produce any significant change in AK activity (Fig. 4 A). The addition of AEC (a lysine analogue) to the assay mixture, although resulting in inhibition of AK activity, was shown to be less effective than lysine alone (Fig. 4 A), whereas SAM was only able to inhibit AK activity synergistically, when present with either lysine or threonine (Fig. 4C).

The effect of these compounds on HSDH activity was also tested. Threonine alone and lysine plus threonine were effective in inhibiting HSDH activity at the highest concentration tested (Fig. 4B). Interestingly, the addition of 5 mM valine to the assay mixture also resulted in

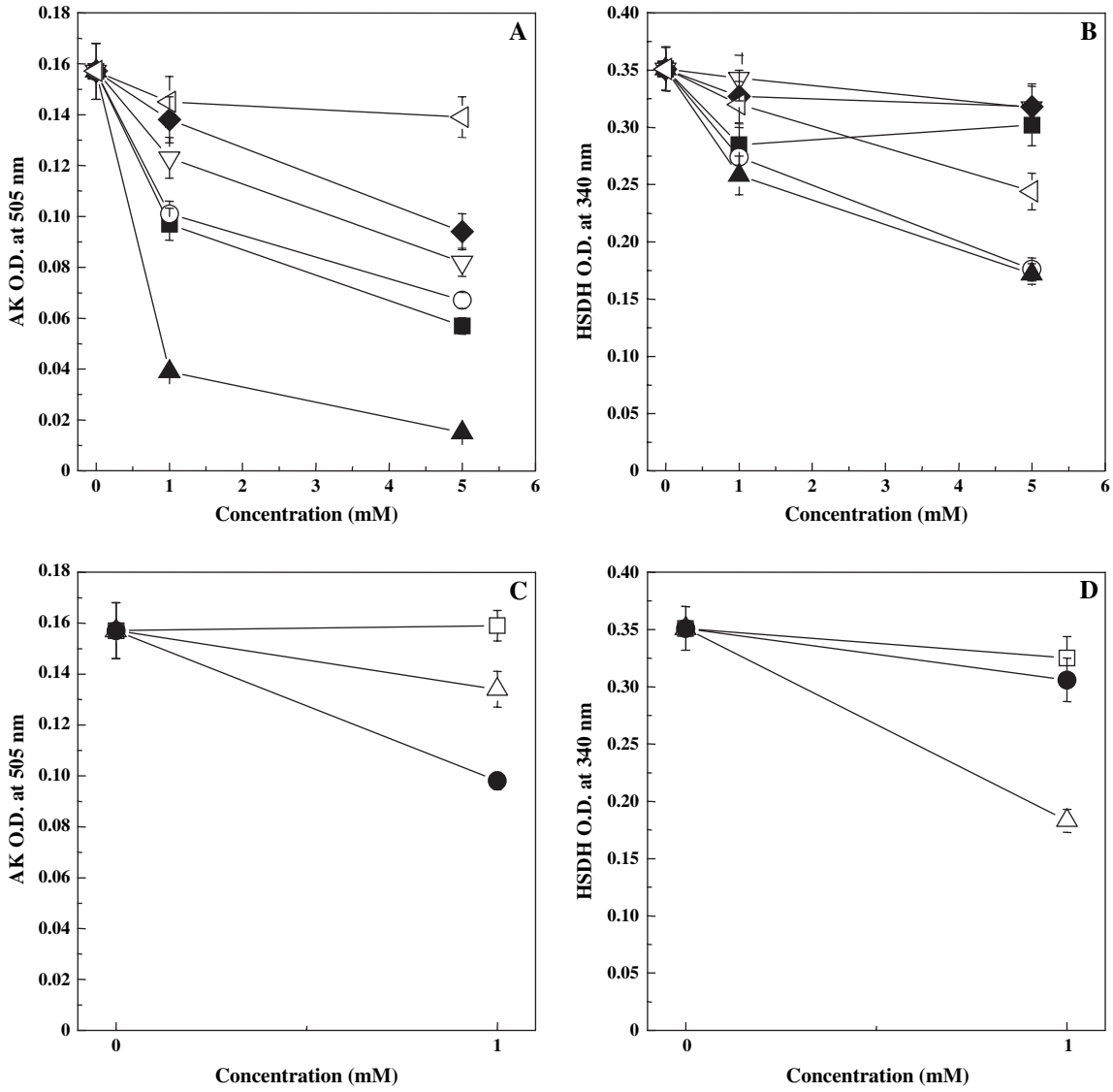

Figure 4 Effect of the addition of amino acids and other compounds on the activities of aspartate kinase (AK) (A, C) and homoserine dehydrogenase (HSDH) (B, D) isolated from sorghum seeds. (■) lysine, (○) threonine, (♦) methionine, (◁) valine, (▽) S-2-aminoethyl-L-cysteine, (▲) lysine plus threonine, (□) S-adenosylmethionine (SAM), (●) SAM plus lysine, (△) SAM plus threonine.

a slight inhibition of HSDH activity, whereas methionine, lysine and AEC had no effect on the enzyme activity (Fig. 4B). The addition of SAM to the HSDH assay did not alter HSDH activity; however, SAM plus threonine resulted in a stronger inhibition of HSDH activity (Fig. 4D).

Effects of calcium, EGTA, calmodulin and compound 48/80 on AK and HSDH activities

The effect of these modulators on the pool of fractions containing AK and HSDH activities eluting from the DEAE-Sephacel column with a 0-500 mM KCl linear gradient was tested (Fig. 5). The AK and HSDH activities did not vary significantly in response to any of the compounds tested (Fig. 5A and Fig. 5B).

Effects of ionic strength on AK and HSDH activities

Similarly, increasing concentrations of KCl did not result in any major alteration in AK (Fig. 5C) or HSDH activity (Fig. 5D), eluting from the DEAE-Sephacel column with a 0–500 mM KCl linear gradient.

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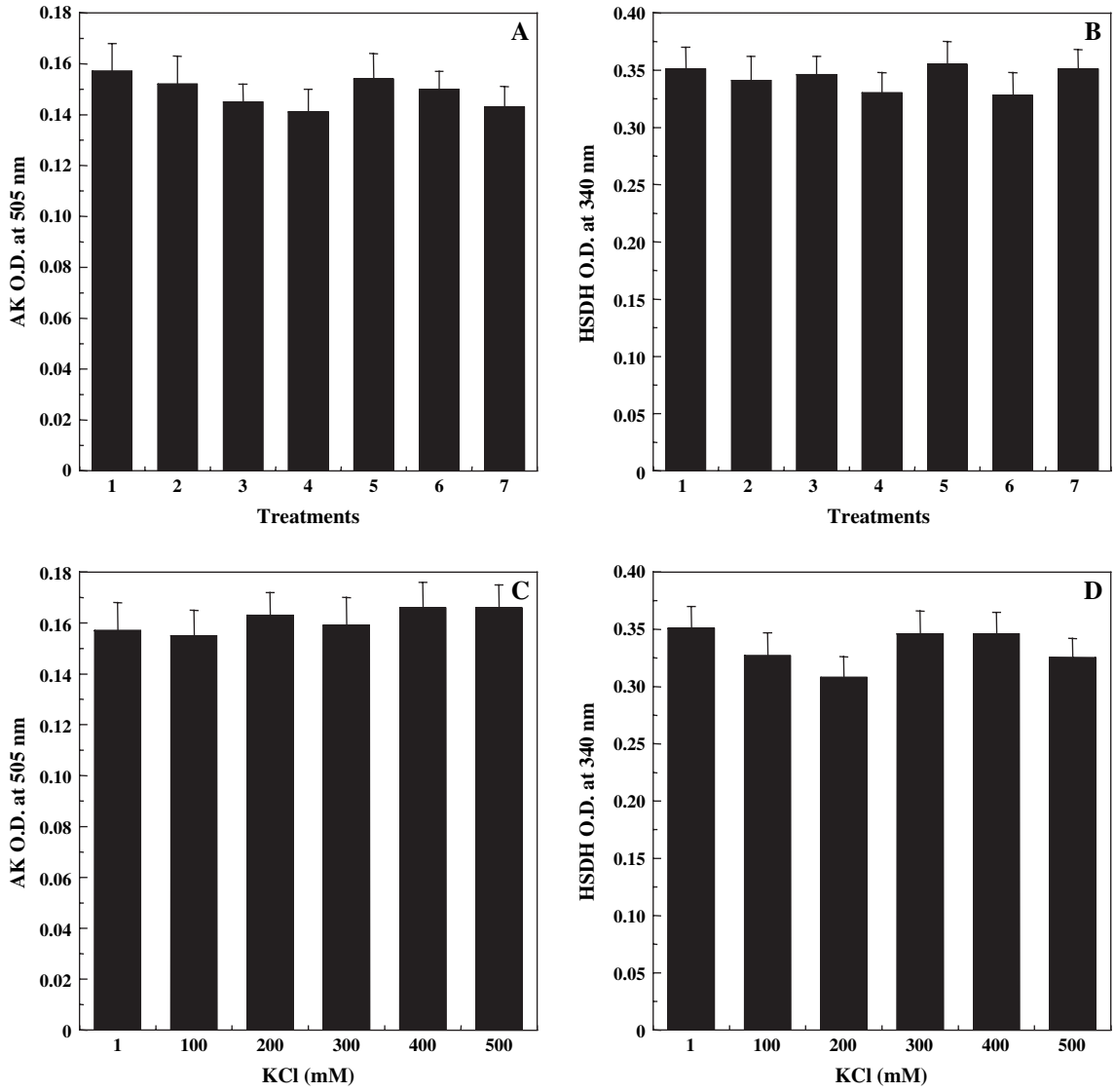

Figure 5 Effect of the addition of distinct compounds on the activities of aspartate kinase (AK) (A, C) and homoserine dehydrogenase (HSDH) (B, D) isolated from sorghum seeds. (1) Control, (2) calcium, (3) ethylene glycol bis (2-aminoethylether)-*N*, *N*, *N′N′*-tetraacetic acid, (4) calcium plus EGTA, (5) calmodulin, (6) calcium plus calmodulin, (7) compound 48/80 and 0–500 mM KCl.

Discussion

AK and HSDH isoenzymes have been purified from several plant species (Azevedo *et al.*, 2006). Anion-exchange and gel filtration chromatography media have been used widely for AK and HSDH purification and shown to produce a good separation of isoenzymes (Azevedo *et al.*, 1997). The purification of both AK and HSDH from immature sorghum seeds in the present study was of a much lower degree, compared to previous studies on other plant species in which similar protein separation techniques were used (Azevedo *et al.*, 1992; Teixeira *et al.*, 1998). Similarly, the recovery was also very low and can only be compared to the result reported for *Coix lacryma-jobi* (coix) seeds, where the enzymes also exhibited very low activity and particularly high instability (Lugli *et al.*, 2002). It would be perhaps useful to test and eventually use polyethylene glycol instead of ammonium sulphate when analysing plant tissues in which low activities are detected, which may help reducing the loss of enzyme activity.

Our data suggest that there are at least two overlapping peaks eluting from the anion-exchange chromatography column used (Fig. 2A). Both peaks were characterised as

lysine-sensitive AK isoenzymes and designated AK-LysI and AK-LysII, based on the inhibitory effect caused by lysine, but a threonine-sensitive AK isoenzyme also appears to exist, co-eluting with the first peak of activity, which was more clearly observed following gel filtration chromatography. According to previous reports, lysine has been widely shown to be the main inhibitor of AK activity in higher plants (Azevedo *et al.*, 1997), the only clear exception being coix seeds, in which the threonine-sensitive AK isoenzyme was predominant (Lugli *et al.*, 2002). The elution profile of AK from the gel filtration column indicated the presence of a first peak of AK activity strongly inhibited by threonine and a second peak of activity of minor intensity, but strongly inhibited by lysine (Fig. 3A). Threonine-sensitive AK isoenzymes have also been identified in plants, when fractions separated by distinct methods were tested with threonine and lysine individually. The results obtained for sorghum confirm the hypothesis that all plants have both lysine- and threonine-sensitive AK isoenzymes, although the number of isoenzymes and relative contributions may vary (Azevedo *et al.*, 2006). The threonine-sensitive AK isoenzyme exhibited a molecular mass of 167 kDa and the lysine-sensitive AK isoenzyme a molecular mass of 79 kDa based on the gel filtration chromatography data. These results are different from those that have been previously reported for some plants in which the molecular mass of the lysine-sensitive AK isoenzymes varied from 104 to 250 kDa, depending on the plant species (Relton *et al.*, 1988).

For sorghum HSDH, two overlapping peaks of activity were eluted from the anion-exchange chromatography column, which could be classified as two distinct isoenzymes, one threonine-resistant HSDH (HSDH-R) and the other a threonine-sensitive HSDH (AK/HSDH-Thr) (Fig. 2B). The existence of both isoenzymes was further confirmed by gel filtration chromatography (Fig. 3B). It has been observed that both isoenzymes of HSDH from plants are unstable and that the threonine-sensitive isoenzyme is easily altered and desensitised (Azevedo *et al.*, 1997). Despite the lack of success in completely separating the isoenzymes, the co-elution of the threonine-sensitive AK and HSDH activities confirmed also for sorghum, the existence of a bifunctional protein containing both threonine-sensitive activities of AK and HSDH (AK/HSDH-Thr) and observed in essentially all plant species tested (Azevedo *et al.*, 1997).

Further analysis of the mixed fractions in the presence of lysine and threonine separately and together confirmed the isoenzyme pattern suggested above for sorghum. Moreover, it was also clear that during the purification procedures, there was a considerable loss in total enzyme activity, which affected the AK isoenzymes differently. After the anion-exchange chromatographic separation, the lysine-sensitive AK activity was largely predominant, a scenario that was completely altered after gel filtration chromatography. A similar result was also observed for HSDH activity with a drastic loss in activity, which led to an altered contribution of the two HSDH isoenzymes, initially dominated by the threonine-resistant isoenzyme (HSDH-R) and later by the threonine-sensitive HSDH isoenzyme (AK/HSDH-Thr). Such a result is particularly important since it is clear that the majority of the AK and HSDH activity at the end of the purification procedure is due mainly to the bifunctional AK/HSDH-Thr polypeptide, which appears to be more stable than the monofunctional isoenzymes of AK (AK-LysI and AK-LysII) and HSDH (HSDH-R).

The addition of AEC did not cause inhibition of AK activity to the same extent as that obtained with lysine. AEC has been shown to be able to substitute for lysine in proteins and also to act as a substrate for LOR in rice, but again not to the same extent as lysine (Gaziola *et al.*, 2000). The effects of methionine and valine on AK and HSDH activities have been investigated previously in other plant species (Lugli *et al.*, 2000). For the sorghum seed enzyme, the addition of methionine to the assay mixture reduced AK activity, whereas valine at a high concentration partially inhibited HSDH, a result that has not been reported in the past. These results are difficult to explain and further investigation is clearly necessary.

It is also well established that calcium plays an important role in plant signalling (Hetherington & Brownlee, 2004). The regulatory action of calcium ranges from control of ion transport to gene expression. Increase in cytosolic calcium concentration in response to extracellular stimuli is considered as the primary event in a range of physiological responses including the modulation of proteins (Reddy & Reddy, 2004). In earlier studies, it was shown that phytochrome activation of AK is mediated through calcium, with further evidence *in vivo* provided when elevated levels of intracellular calcium participate in the activation of AK in immature pods of *Cicer* (Dey & Guha-Kukherjee, 2000). However, such a regulatory role for calcium on AK could not be confirmed in a series of other reports with different plant species (Azevedo *et al.*, 1997). This is now also valid for AK and HSDH isolated from sorghum seeds, since neither of the enzyme activities were affected by any of the treatments tested, indicating that calcium does not regulate AK and HSDH activity *in vitro.* Although a controversy exists about the regulatory role of calcium in lysine biosynthesis in plants, a regulation of the enzymes involved in lysine catabolism has definitely been confirmed (Kemper *et al.*, 1998; Gaziola *et al.*, 2000; Zhu *et al.*, 2002).

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Finally, it is important to note that the sorghum line used by our group in this study and in other studies on lysine metabolism had never been characterised for lysine content of the seed. Interestingly, a group of high-lysine sorghum lines is now available from the International Crops Research Institute for the Semi-Arid Tropics, IS 11758, IS 11167, IS 16210, IS 10477, IS 22204, IS 5603 and also the HPD/hl sorghum mutant very recently reported (Tesso *et al.*, 2006). Notwithstanding, the Massa 03 sorghum line used in this study exhibited total (soluble and protein) seed lysine concentrations at levels identical to that of the IS lines listed above (Fornazier *et al.*, 2005). High-lysine mutant lines, such as those isolated for maize, have been shown to have altered regulation of the enzymes involved in lysine metabolism (Azevedo *et al.*, 2003, 2004*a*,*b*), and others have been produced by genetic manipulation of lysine biosynthesis and storage proteins (Huang *et al.*, 2004, 2005). Therefore, two scenarios must be considered when sorghum is concerned: (a) the high lysine concentration observed in the seeds of all sorghum lines reported in the literature may well be considered normal for this plant species and (b) the regulatory properties of AK and HSDH, such as those determined for the LOR and SDH enzymes, must be taken into account.

Acknowledgments

This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant 01/13216-4 and 04/16039-4) and the British Council. R. A. A. and R. R. F. also thank CNPq and FAPESP (00/05960-2), respectively, for the fellowship and scholarship granted.

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